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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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## PRIORITY DOCUMENT

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Sheet 2 of the certificate  
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**EUROSCREEN S.A.**  
**1160 Bruxelles**  
**BELGIUM**

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Titre de l'invention:

**High-throughput screening diagnostic and/or dosage method of an agonist and/or an antagonist for a calcium-coupled receptor**

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5

10     HIGH-THROUGHPUT SCREENING DIAGNOSTIC AND/OR DOSAGE METHOD  
      OF AN AGONIST AND/OR AN ANTAGONIST FOR A CALCIUM-COUPLED  
          RECEPTOR

Field of the invention

      The present invention is related to a high-  
15 throughput screening diagnostic and/or dosage method and  
device of an agonist and/or an antagonist for a calcium-  
coupled receptor and the agonist and/or antagonist of said  
calcium-coupled receptor identified by said method and  
device.

20

Background of the invention and state of the art

      A lot of G-protein-coupled receptors (GPCR)  
trigger, upon binding of an agonist, a transient increase  
in intracellular calcium concentration. This variation acts  
25 as an internal secondary messenger and is an important  
modulator of many physiological mechanisms (reviewed by  
Rink (1990), Tsunoda (1993) and by Santella & Carafoli  
(1997)). Measurement of intracellular calcium concentration  
in cells expressing a GPCR can thus be used to monitor the  
30 efficacy of activation of a GPCR by various compounds known  
- or suspected - to be a ligand for this GPCR.

      Changes in calcium concentration can be  
detected by several means and methods, like the use of  
fluorescent dyes (for example: fura-2, fluo-3 and indo-1).

However,  $\text{Ca}^{++}$  sensitive dyes have limitations. Activation of the dyes with an excitation beam requires complicated and expensive instruments and limits the use of the plastic labware such as microtiter plates.

5 Another method for intracellular calcium concentration measurement is the use of cell lines overexpressing a GPCR and apoaeguorin, such as described by Sheu et al (1993). In this system, cells expressing apoaeguorin are incubated with coelenterazine, which is the  
10 co-factor of aequorin. During this incubation, coelenterazine enters the cell and conjugates with apoaeguorin to form aequorin, which is the active form of the enzyme. Upon incubation of the cells with an agonist of the GPCR, intracellular calcium concentration increases.  
15 This increase leads to the activation of the catalytic activity of aequorin, which oxidises coelenterazine and yields apoaeguorin, coelenteramide,  $\text{CO}_2$  and light. Once the photon has been emitted, the complex must dissociate and apoaeguorin must recombine with a new coelenterazine  
20 molecule to be able to emit light again. Thus, in this system, measurement of light emission following agonist addition reflects its ability to activate the GPCR and thus to increase intracellular calcium concentration. Because light is emitted only during 20 to 30 seconds after  
25 activation of the GPCR, recording of the emitted light must be performed during the few seconds following agonist addition to the cells. This flash-type signal is due to the fact that (1) intracellular calcium increase triggered by GPCR is only transient and (2) as mentioned earlier, after  
30 oxidation of coelenterazine, apoaeguorin must recombine with coelenterazine to be able to emit light again.

The Patent Application EP-0341477 teaches the expression of jellyfish photoprotein aequorin in a

mammalian cell system by cloning gene pAQ440 specifying the biosynthesis of the aequorin into an expression vector plasmid of a mammalian cell system, subjecting the resulting plasmid to transfection and producing the photoprotein aequorin in the mammalian cell.

The Patent US-5,422,266 describes a gene encoding apoaequorin protein included in a vector capable of expressing the apoaequorin in a micro-organism such as E. coli.

10 The Patent US-5,714,666 describes mammalian cell lines or transgenic animals expressing apoaequorin and a receptor involved in the modulation of intracellular calcium. This document also describes a method of measuring intracellular calcium comprising adding coelenterazine  
15 cofactors to said mammalian cells expressing apoaequorin and measuring photoemission where emission of photons is indicative of intracellular calcium concentration.

However, the methods of the state of the art require firstly the spreading of cells from a mammalian  
20 cell line expressing apoaequorin on a solid support (for example a 96-well plate), secondly the addition of the coelenterazine cofactor upon the cells and incubation to reconstitute a functional aequorin, thirdly the preparation of the agent affecting a receptor involved in the  
25 modulation of intracellular calcium concentration, and its addition to the prepared cells, and finally the measurement of the photoemission.

Furthermore, as mentioned above, light is emitted only during 20 to 30 seconds after activation of  
30 the GPCR. Therefore, the recording of the emitted light must be performed during the few seconds following agonist addition to the cells.

Therefore, the methods used in the state of the art are not adequate for a detection based upon high-

throughput screening level, which usually need luminometer(s) and require the use of microtiter plates for the testing of thousands of compounds.

5 Aims of the invention

The present invention aims to provide a method and means, which do not present the drawbacks of the state of the art, for detecting biologically active substances, especially agonists and/or antagonists for  
10 calcium-coupled receptors.

A main aim of the present invention is to provide such method and means which allow the detection of biological active substances at a high-throughput scale, which could be adapted to microtiter plates without  
15 requiring the modification of the high-throughput screening device.

Another aim of the present invention is to provide an easy and non-expensive method which could be easily automated.

20

Summary of the invention

The present invention is related to a high-throughput screening diagnostic and/or dosage method of an agonist and/or an antagonist (or a reverse agonist) for a  
25 calcium-coupled receptor, comprising the following successive steps :

- disposing the agonist and/or the antagonist upon a solid support,
- incubating cells expressing apoaeguorin and said  
30 calcium-coupled receptor with coelenterazine in order to reconstitute an active aequorin by said cells,
- adding to said solid support said cells, and



- obtaining the measurement of an emitted light by said cells.

Advantageously, the solid support is a microtiter plate, preferably a 96-well microtiter plate.

5 Advantageously, cells expressing apoaeguorin and the calcium-coupled receptor are cells expressing G-coupled receptor and possibly one or more protein(s) intended to ensure a coupling of the receptor to the calcium pathway.

10 Preferably, said protein is selected from the group consisting of a natural G $\alpha$ 16 protein, a chimeric G-protein resulting from a fusion between two different G-proteins or a phospholipase C $\beta$ 2 protein.

The measurement of the emitted light is  
15 advantageously obtained with one or several luminometer(s), possibly equipped with several dispensers and measurement heads.

The present invention is also related to a high-throughput screening diagnostic and/or dosage device  
20 intended for the diagnostic and/or dosage method according to the invention, said device comprising the following elements :

- a microtiter plate, preferably a 96-well microtiter plate,
- 25 - a medium containing cells expressing apoaeguorin and a calcium-coupled receptor,
- a medium containing coelenterazine, and
- means, such as one or more luminometer(s) equipped with one or several dispensers and measurement heads, for  
30 detecting an emitted light by said cells.

Advantageously, the device according to the invention comprises means for the automatic performance of

the successive steps of the diagnostic and/or dosage method according to the invention.

A last aspect of the invention is related to the agonist and/or the antagonist of a calcium-coupled  
5 receptor identified by the method or the device according to the invention.

The present invention will be described in details in the following non-limiting examples, in reference to the enclosed figures.

10

Brief description of the drawings

Figure 1 shows a series curves representing the intensity of the emitted light by cells as a function of time for each well of a 96-well  
15 plate injected with cells. The scaling is the same for all the graphs. Recording of the signal was performed for 30 seconds. Ligand concentrations are increasing from column 1 towards column 12. All measurements were performed in duplicate : lines A and B :  
20 ligand is RANTES; lines C and D : ligand is MIP1- $\alpha$ ; lines E and F : ligand is MIP1- $\beta$ ; lines G and H : ligand is derivative A of RANTES.

25 Figure 2 represents the dose-response curve for different agonists of the CCR-5 receptor which represent RLU (integration of emitted light on 30 seconds) according to the logarithm of the final concentration of the  
30 ligand.

Figure 3 represents the dose-response curve for different agonists of the 5HT-2B receptor.

Figure 4 represents the dose-response curve of the light emitted from CCR-3 aequorin K562 cells as a response to the activation of the receptor by eotaxin.

5 Figure 5 represents the dose-response curves for cells expressing aequorin and G $\alpha$ 16 and (panel A) the MC4 receptor or (panel B) the CB1 receptor.

10 Figure 6 represents the stability of light emitted by coelenterazine-loaded cells maintained in suspension (measurement of RLU (integration on 30 seconds) according to the time (hours)).

15 Description of a preferred embodiment of the present invention

Detection of agonistic activities by means of mammalian cell lines expressing apoaequorin and a GPCR requires the measurement of the emitted light to be  
20 performed just after placing the cells in contact with the supposed agonist. This can easily be measured at low throughput using a single-tube luminometer. However, up to now, this biological system could not be used at a high-throughput scale. Indeed :

25 (1) the necessity to measure light just after placing the cells in contact with the agonist to be tested compels to use a luminometer equipped with a build-in dispenser. For example, due to the short duration of light emission, it is impossible to inject the drugs  
30 to be tested on the cells placed in the 96 wells while the plate is outside the luminometer and to subsequently record emitted light with the plate into the luminometer. Even if the plate could rapidly (i.e.

in less than 15 seconds) be placed into the luminometer after injection of the drugs to be tested, current apparatus do not allow the measurement of light from the 96 wells before the extinction of the flash signal of aequorin, as these luminometers are not equipped with 96 detectors.

- (2) luminometers equipped with a build-in dispenser only allow to inject a single solution into the 96 wells, making it impossible to inject a different drug in each well. Moreover, the washing of the dispenser before each measurement, for the injection of another drug in the next well, is time-consuming and thus is not suitable for the high-throughput scale. The same problem occurs with devices equipped with 6 dispensers (e.g. the "Microbeta Jet" from EG&G Wallac) as the 6 dispensers only deliver a single solution.

The present invention provides a method for performing high-throughput screening of drugs binding to GPCR by the use of mammalian cell lines expressing apoaequorin and a GPCR and by the use of a conventional luminometer. Following this method, the solutions to be tested for (ant)agonistic activities are placed in the wells of a 96-well plate. Cells expressing apoaequorin and a GPCR are detached from the culture plate (if not cells cultured in suspension) and are incubated with coelenterazine to reconstitute active aequorin. These are then maintained in suspension with a magnetic stirrer and the cell suspension is injected, well by well, on the solutions of supposed agonist to be tested. Light emission is then recorded for 1 (alternatively up to 30) seconds. This method, by injecting the same cell suspension in each of the 96 wells, avoids the need of washing the dispenser between each measurement and allows to perform 96 measurements of agonist-induced aequorin light emission in

15 minutes or less with a single dispenser luminometer. Alternatively, it allows to perform 96 measurements of agonist-induced aequorin light emission in 2 minutes or less with a luminometer equipped with 6 dispensers and 5 measurement heads (e.g. with the "Microbeta Jet" from EG&G Wallac).

This method thus allows to perform high-throughput screening (10 000 samples/day) with mammalian cell lines expressing apoaequorin and a GPCR and by the use of a conventional luminometer. This reduces the screening time and the amount of drugs needed for each measurement.

This system also allows to perform a functional screening with very few (down to 5000) cells per measurement.

15 The injection of the cells into the wells containing the agonists did not high the background of the measurement (that could for example have originated from cell breakage, letting some aequorin molecules come from the cells into the culture medium, where the calcium concentration would have triggered the emission of light from aequorin). A signal-to-noise ratio above 50 was commonly obtained with this system of cell injection.

The method according to the invention is suitable for performing high-throughput analysis of GPCR or other calcium-coupled-receptor stimulation by known or supposed agonists by means of cells expressing the receptor and apoaequorin. These cells may express apoaequorin in the cytoplasm, as described by Sheu et al. (1993) or Button and Brownstein (1993) or may express apoaequorin in the mitochondria, by means of the addition of a mitochondrial targeting sequence to the aequorin, as used by Stables et al. (1997) or in any other part of the cell. These cells may also express proteins intended to ensure coupling of the over-expressed receptor to the calcium pathway. These

may be the natural G $\alpha$ 16 protein (Milligan et al., 1996), chimeric G proteins resulting from a fusion between two different G proteins (Komatsuzaki et al., 1997), phospholipase C- $\beta$ 2 (Park et al., 1992), or any other  
5 "universal coupling" protein.

### Examples

#### Example 1

A CHO cell line expressing the chemokine CCR-  
10 5 receptor, the G $\alpha$ 16 coupling protein and apoaeguorin was established. Cells were cultivated as a monolayer in HAM'sF12 medium containing 10% Foetal bovine serum (FBS). On the day of the experiment, the culture medium was removed and cells were incubated for 5 min at room  
15 temperature in PBS-EDTA (phosphate buffered saline solution without calcium, added with 5 mM EDTA). Cells were detached from the culture vessel by shaking the culture plate by hand and by pipetting up and down. Cells were centrifuged and the supernatant was removed to eliminate the EDTA; the  
20 pellet was resuspended in culture medium HAM'sF12 without FBS and with 0.1% Bovine Serum Albumin. Cells were counted by means of a Thomas cell, were centrifuged again and were resuspended in culture medium HAM'sF12 without FBS and with  
25 0.1% Bovine Serum Albumin at a concentration of  $5 \cdot 10^6$  cells/ml. Coelenterazine (or a derivative of it, e.g. Coelenterazines f, h, n, cp or hcp, from Molecular Probes Inc.) at 500  $\mu$ M in methanol was added to the cell suspension at a final concentration of 5  $\mu$ M. The cell suspension was then stored in the dark at room temperature  
30 for 3 to 5 h, with shaking every 15 to 30 min to maintain the cells in suspension.

Series of dilutions of known ligands were prepared in medium HAM'sF12 without FBS and with 0.1%

Bovine Serum Albumin and 50  $\mu$ l of each of these solutions were placed in the wells of a 96-well plate. The cell suspension was diluted 5 times with medium HAM'sF12 without FBS and with 0.1% Bovine Serum Albumin and was placed in a glass or plastic container protected from light by wrapping it with aluminium paper. A magnetic stirring bar was added to the suspension and a magnetic stirrer was used at low speed (1 to 5 rounds per second) to maintain the cells in an homogenous suspension. The magnetic stirring bar was equipped with a ring to avoid to crush cells, and to avoid subsequently the release of aequorin in the culture medium. Alternatively, a culture vessel equipped for culture of cells in suspension may be used.

One uses the EG&G Wallac's MicroLumat-Plus microplate luminometer, which allows injection and direct subsequent recording of the emitted light from each well of a 96-well plate. The end of the entrance tube of the dispenser was placed at the bottom of the cell suspension and the dispenser was washed 3 times the dead volume of the apparatus so that all the volume of the tube and pumps was filled with the cell suspension. The 96-well plate containing the solutions of agonists was then inserted into the luminometer. Then, for each well, 50  $\mu$ l of the cell suspension (i.e. 100 000 cells) was dispensed into the well (at the lowest injection speed (0.4 s) to prevent cell breakage that would release aequorin into the culture medium) and the emitted light was immediately recorded during 30 seconds. After reading the first well, cells were injected into the next well and emitted light was recorded, etc. For each plate, a series of curves representing the intensity of the emitted light as a function of time for each well was displayed (figure 1). The intensity of the emitted light was integrated over 30 s using the Winglow software provided with the luminometer, yielding for each

well one value representative of the emitted light and hence of the stimulation of the CCR-5 receptor by the agonist present in the well. These values can be plotted against the logarithm of the ligand concentration to  
5 generate dose-response curve as shown in figure 2. These allow the determination of half-maximal response doses ( $EC_{50}$ ) for each ligand. For the generation of these data, 288 measurements were performed in less than 3 hours.

10 Example 2

A CHO cell line expressing the serotonin 5HT-2B receptor, the  $G\alpha_{16}$  coupling protein and apoaeguorin was established. Cells were treated as described in Example 1 and were dispensed after dilution (100  $\mu$ l/well,  
15 corresponding to 50 000 cells) on 100  $\mu$ l of solutions of known agonists for this receptor. The emitted light was recorded during 20 s for each well. Dose-response curves obtained for different agonists are shown in figure 3. For the generation of these data, 144 measurements were  
20 performed in less than 1 hour.

Example 3

K562 cells expressing the chemokine CCR3 receptor were transfected by a plasmid for the expression  
25 of aequorin and the  $G\alpha_{16}$  coupling. Cells stably transfected were selected for 2 weeks with the antibiotic Zeocin. These cells were culture in suspension in DMEM culture medium containing 10% FBS. They were centrifuged and the pellet was used as described in example 1 to perform aequorin  
30 measurements. A dose-response curve with eotaxin generated by this method is described in figure 4.



Example 4

A CHO cell line expressing the melanocortin MC4 receptor, the G $\alpha$ 16 coupling protein and apoaeguorin was established. Cells were treated as described in Example 1 and were dispensed after dilution (100  $\mu$ l/well, corresponding to 50 000 cells) on 100  $\mu$ l of solutions of a known agonist for this receptor. The emitted light was recorded during 20 s for each well. A dose-response curve obtained with this method is showed in figure 5 A.

Example 5

A CHO cell line expressing the cannabinoid CB1 receptor, the G $\alpha$ 16 coupling protein and apoaeguorin was established. Cells were treated as described in Example 1 and were dispensed after dilution (100  $\mu$ l/well, corresponding to 50 000 cells) on 100  $\mu$ l of solutions of a known agonist for this receptor. The emitted light was recorded during 20 s for each well. A dose-response curve obtained with this method is showed in figure 5 B.

Example 6

A CHO cell line expressing the serotonin 5HT-2B receptor, the G $\alpha$ 16 coupling protein and apoaeguorin was established. Cells were treated as described in Example 1 and, every 15 min, cells were dispensed (100  $\mu$ l/well, corresponding to 50 000 cells) on 100  $\mu$ l of a single solution (same concentration in all the wells) of a known agonist for this receptor. The emitted light was recorded during 30 min for each well, after that time, the next well was measured in the same way. The intensity of the light emitted over the first 30 s was integrated for each well. The resulting values were plotted as a function of time (figure 6), showing that, after loading with

14

coelenterazine, the cells can be maintained in suspension and used during at least 15 h for the measurement of agonistic activities.

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5 crosstalk in stimulus secretion coupling. Biochim. Biophys.  
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CLAIMS

1. Diagnostic and/or dosage method of an agonist and/or an antagonist for a calcium-coupled receptor, comprising the following successive steps :

- 5 - disposing the agonist and/or the antagonist upon a solid support,  
- incubating cells expressing apoaeguorin and said calcium-coupled receptor with coelenterazine in order to reconstitute an active aequorin by said cells,  
10 - adding to said solid support said cells, and  
- obtaining the measurement of an emitted light by said cells.

2. Method according to claim 1, wherein the solid support is a microtiter plate.

- 15 3. Method according to claim 2, characterised in that the microtiter plate is a 96-well microtiter plate.

4. Method according to any one of the preceding claims, characterised in that the cells express apoaeguorin in the cytoplasm or in the mitochondria or in  
20 any other part of the cell.

5. Method according to any one of the preceding claims, wherein cells expressing a calcium-coupled receptor are cells expressing G-protein-coupled receptor and/or cells which express proteins intended to  
25 ensure a coupling of the analysed receptor (endogenous or overexpressed) to the calcium pathway.

6. Method according to claim 5, wherein said protein is selected from the group consisting of natural G $\alpha$ 16 protein, chimeric G-protein resulting from a fusion  
30 between two different G-proteins or phospholipase C $\beta$ 2 protein or any other coupling protein or chemical.

7. Method according to any one of the preceding claims, characterised in that the measurement of

the emitted light is obtained with one or more luminometer(s), advantageously equipped with several dispensers and measurement heads.

8. High-throughput screening diagnostic  
5 and/or dosage device intended for the high-throughput screening diagnostic and/or dosage method according to any one of the preceding claims, comprising the following elements :

- 10 - a microtiter plate, preferably a 96-well microtiter plate,
- a medium containing cells expressing apoaeguorin and a calcium-coupled receptor,
- a medium containing coelenterazine, and
- means for detecting an emitted light by said cell(s).

15 9. Device according to claim 8, comprising means for the automatic performance of the successive steps of the diagnostic and/or dosage method according to any one of the claims 1 to 7.

20 10. Agonist or antagonist of a receptor identified by the method according to any one of the claims 1 to 8.

ABSTRACT

HIGH-THROUGHPUT SCREENING DIAGNOSTIC AND/OR DOSAGE METHOD  
OF AN AGONIST AND/OR AN ANTAGONIST FOR A CALCIUM-COUPLED  
RECEPTOR

5

The present invention is related to a diagnostic and/or dosage method of an agonist and/or an antagonist for a calcium-coupled receptor, comprising the following successive steps :

- disposing the agonist and/or the antagonist upon a solid support,
- incubating cells expressing apoaeguorin and said calcium-coupled receptor with coelenterazine in order to reconstitute an active aequorin in said cells,
- adding to said solid support said cells, and
- obtaining the measurement of an emitted light by said cells.

20

The present invention is also related to the diagnostic and/or dosage device intended for the method according to the invention.

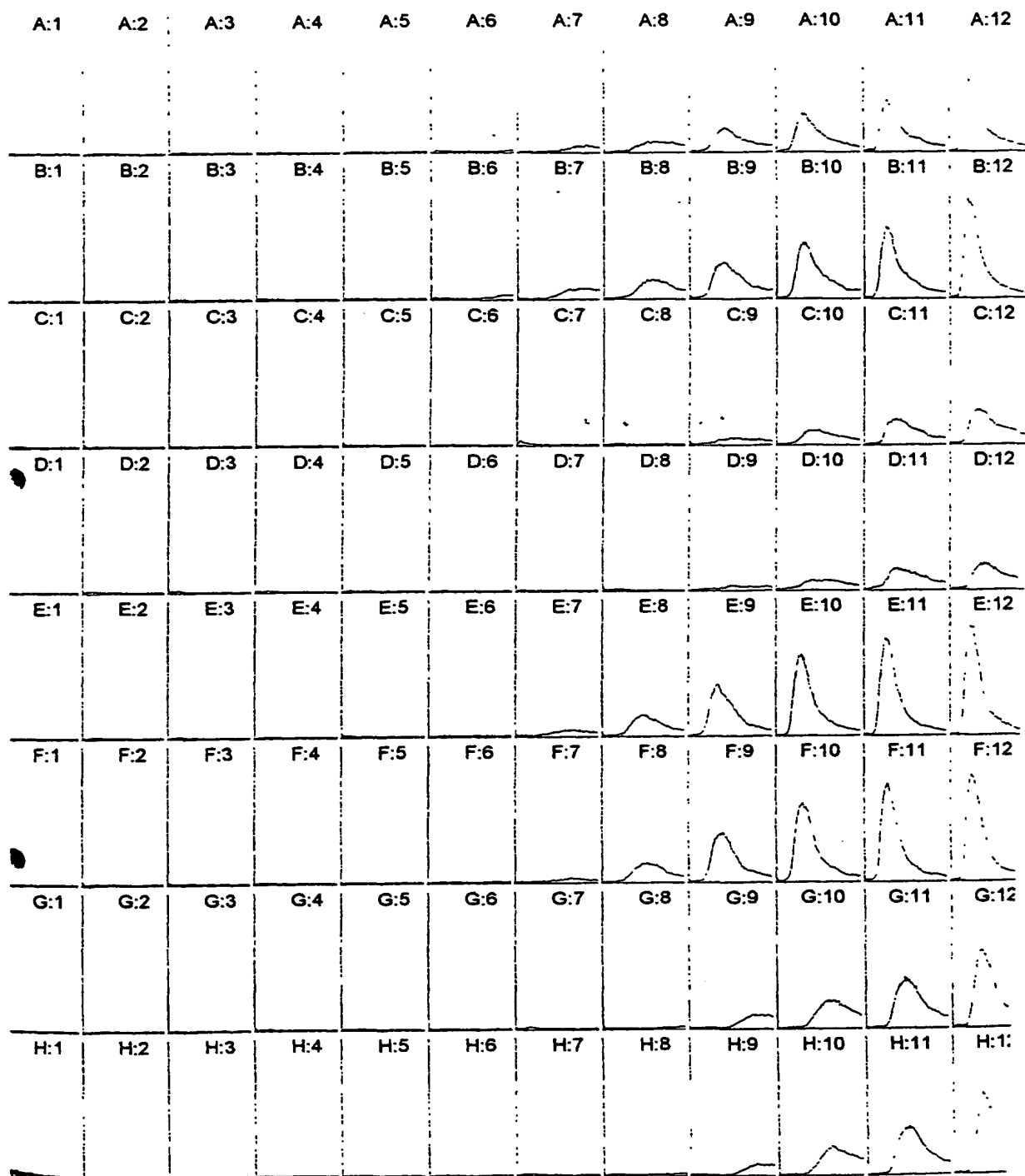
(Figure 2)





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FIG. 1



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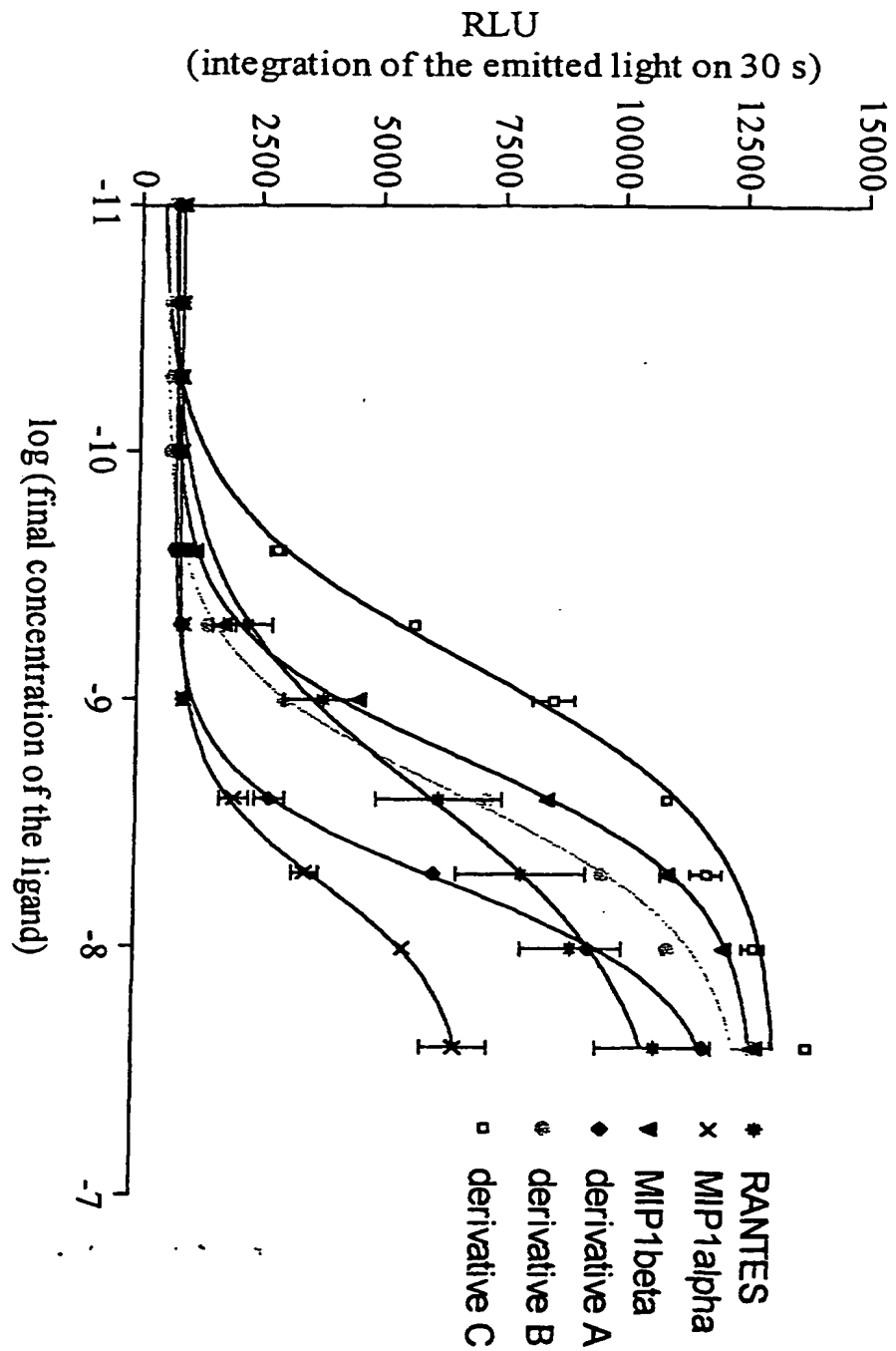


FIG. 2

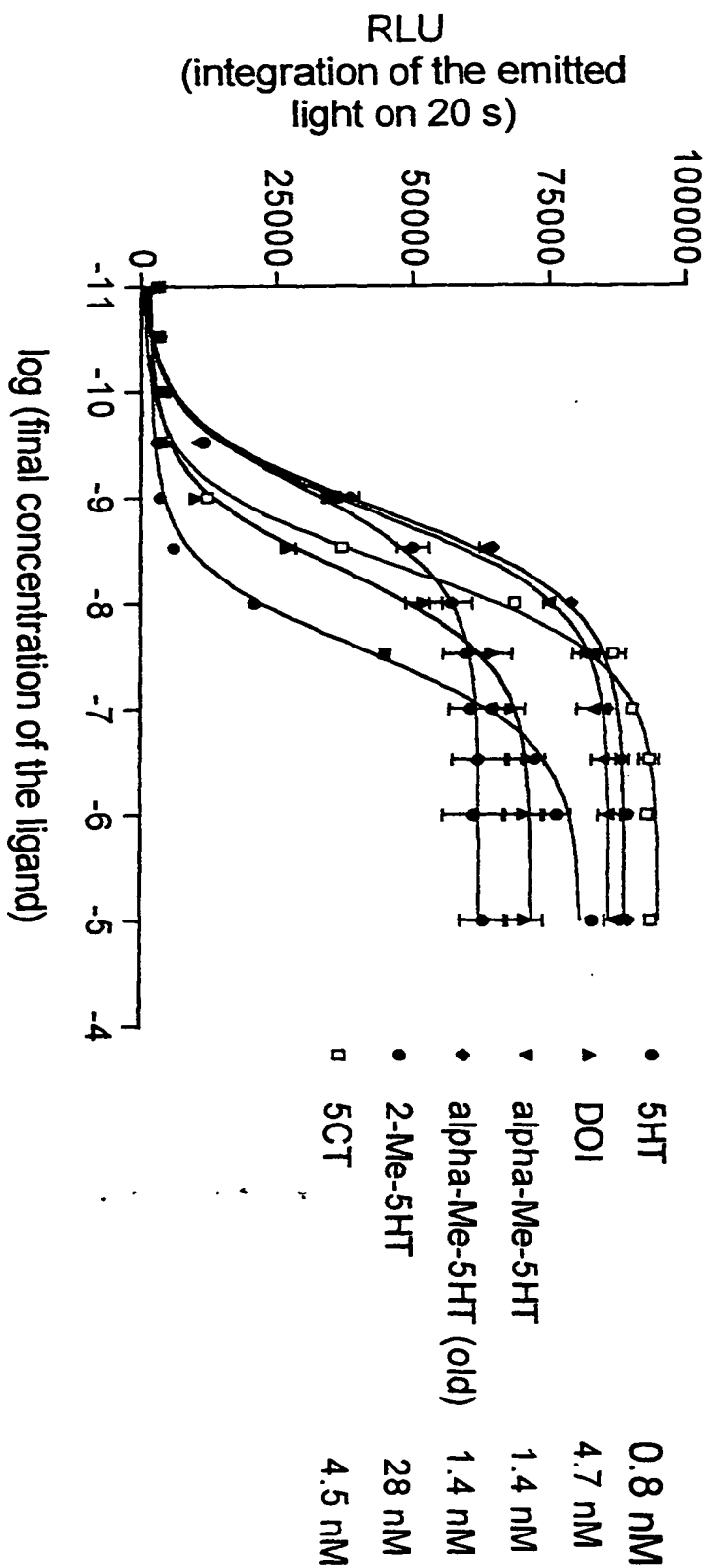
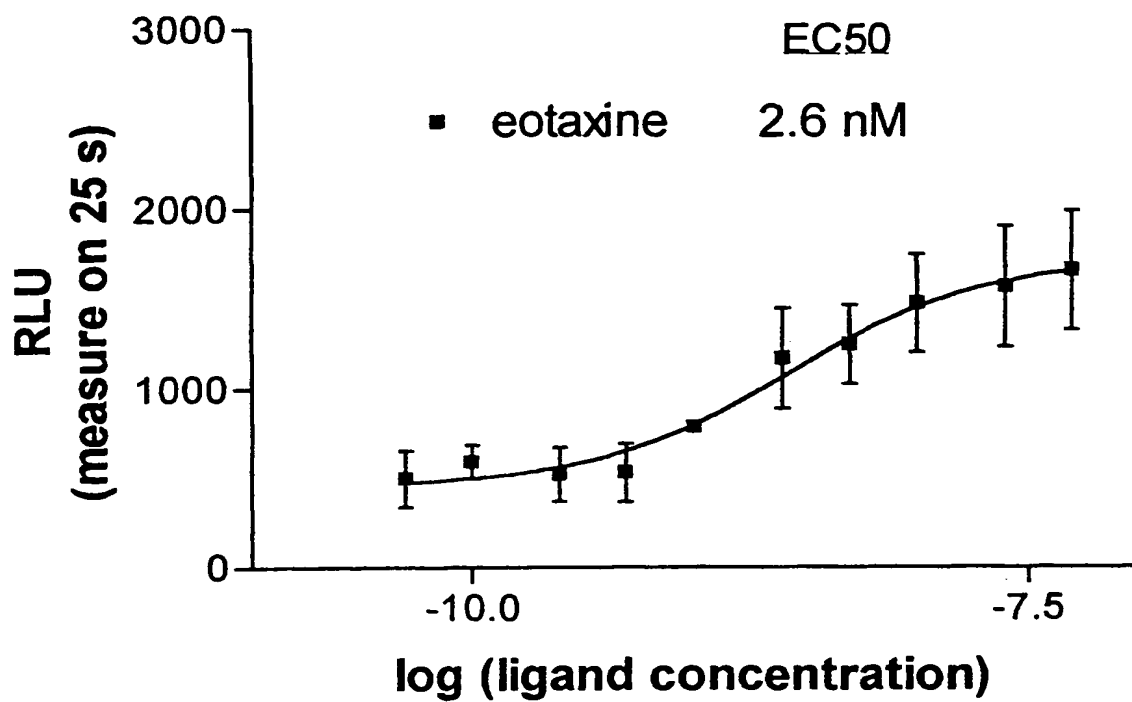


FIG. 3

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FIG. 4

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Cellules CHO-MC4R-G $\alpha$ 16-aequorine

FIG.5A

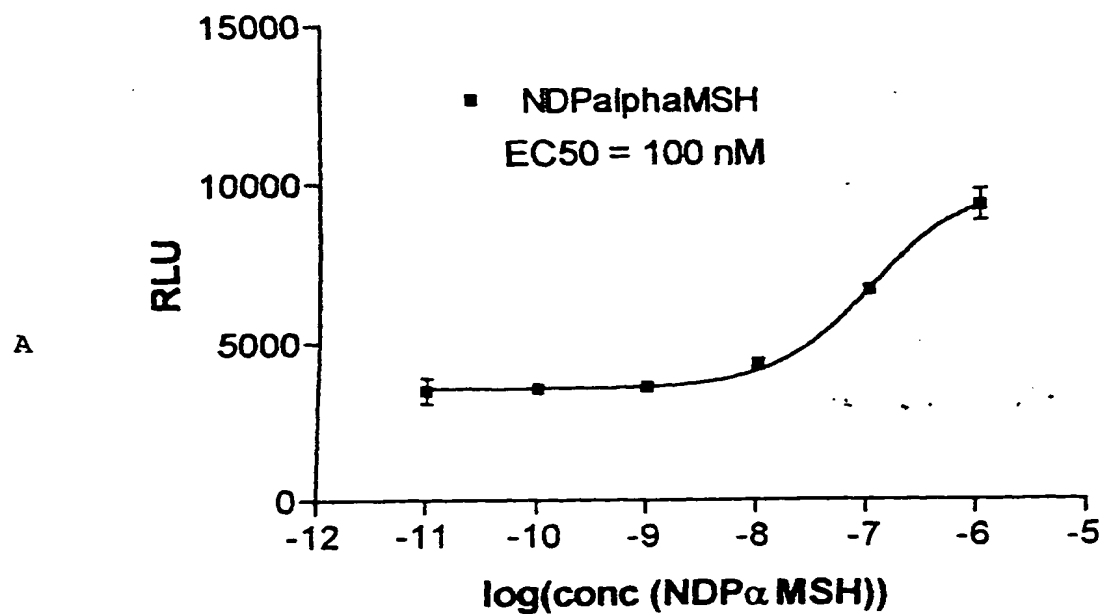
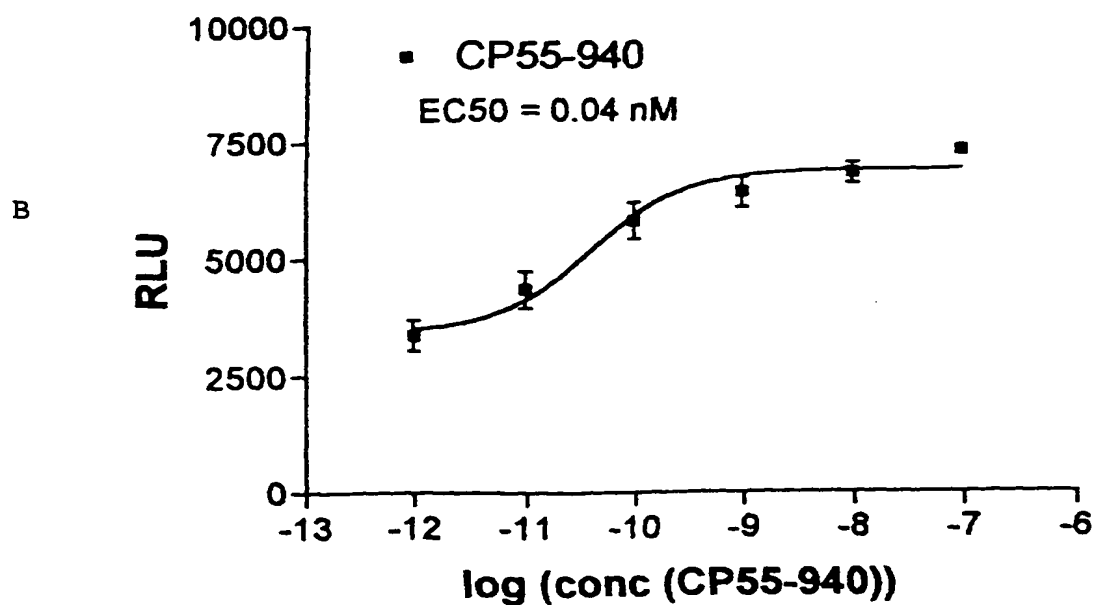
Cellules CHO-CB1-G $\alpha$ 16-aequorine

FIG.5B



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